A STUDY OF THE FACTORS WHICH INTERFERE WITH THE USE OF YEAST AS A TEST ORGANISM FOR THE ANTINEURITIC SUBSTANCE

Geraldo de Paula Souza

Submitted in partial fulfilment of the requirements for the Degree of Doctor of Public Health, School of Hygiene and Public Health

Johns Hopkins University

June
1920

In a recent paper Williams* described a method of testing for the presence of the antineuritic substance which in this laboratory has been generally referred to as water-soluble B. For this purpose yeast was employed, and the basis of the test was the assumption, verified by experimental trials by Williams, that the yeast cell is capable of multiplication only when the antineuritic substance is present in its nutrient medium. By means of a series of dilutions of solutions which he used for the cultivation of yeast he was able to obtain results which gave promise of his method being useful in a quantitative sense as well as qualitative. Owing to the fact that the test as described by Williams could be carried out with very small amounts of material, and in a period of time much shorter than is required for the demonstration of the presence of the antineuritic substance in a nutrition experiment on a mammal, the method proposed for utilizing yeast as an experimental organism seemed to offer great possibilities of usefulness to those who are engaged in the study of methods for the isolation and identification of this most interesting dietary factor.

The literature relating to yeasts contains numerous references to experiments directed toward the cultivation of this organism in solutions containing the necessary inorganic salts in a state of moderate purity, together with sugar as a source of energy. It is a well established fact that yeast can under appropriate conditions utilize the nitrogen of ammonium salts for the synthesis of the proteins required in the multiplication of its cells. Considerable difference of opinion has existed among investigators as to the readiness with

---

* Williams, R. J. Jour. Biol. Chem., xxxviii, 1919, 465.

a

which yeast can subsist on such medium. According to the interpretation of Williams, based on the earlier work of others, the multiplication of the yeast cell in a medium consisting of purified food-stuffs depended on the size of the mass of yeast used for the inoculation. If but very few cells were introduced they would fail to develop because of the lack of the antineuritic substance, or Funk's "vitamine". If, on the other hand, a fairly large group of yeast cells were introduced into a medium free from the antineuritic substance, a certain proportion of these cells would die and undergo autolysis, and thus contribute to the medium a sufficient amount of the "vitamine" to serve for the nutrition of the remaining cells, whereupon these would undergo division and could produce fermentation, and consequently afford a successful termination of the experiment.

In his earlier experiments Williams used commercial compressed yeast, later he used pure cultures of baker's yeast obtained from the Fleishman Company.

The essential features of the method proposed by Williams are as follows: A suspension of yeast cells in water was obtained for the purpose of securing material for the inoculation of the nutrient medium. The composition of this nutrient medium was as follows:

Cane sugar	20.00 grams
Ammonium sulphate	3.00 "
Potassium phosphate	2.00 "
Calcium chloride	.25 "
Magnesium sulphate	.25 "

This solution was sterilized at 10 lbs. pressure for 10 minutes, and then kept in a refrigerator until ready for use. In Williams' experience the media must be kept without contamination with bacteria, since if it were sterilized after contamination the yeast would develop better than if no organisms had grown in the solution. This presumably was to be interpreted as indicating

that the bacteria furnished a certain amount of the antineuritic substance essential for the growth of yeast. Pacini and Russell* have asserted on the basis of their studies that the typhoid bacillus elaborates a "vitamine" during development.

The test was carried out as follows: A suspension of yeast cells was obtained by gentle shaking or by blowing air through a capillary pipette protected with a cotton plug. 1 cc of the suspension was put into each of the solutions to be tested for the antineuritic substance. As a control 1 tube of sterilized water was also treated with a like amount of the yeast suspension. From each of these tubes after gentle shaking 25 drops were put on a cover slip by means of a sterile writing pen. The cover slip was immediately inverted on a hollow ground slide and sealed with vaseline. If observation with a microscope showed that the drops were too heavily seeded with yeast a new cover slide was prepared, and 25 drops placed upon it with a writing pen after the contents of the tube were diluted sufficiently to reduce to the desired number the yeast cells which would be contained in each of the tiny droplets on the cover slip. At least 50 hanging drops were secured from each solution, these being distributed on two slides. Evaporation was kept in check by cooling the solutions. A record was now made of the number of yeast cells in each square in each drop on the cover slip, and the slides then placed in an incubator at 30° C. They were examined after 6 hours, and a record made of the extent of development of the yeast. Counts were made after 20 to 24 hours incubation. All colonies in which debris from the pen were found were excluded from the countings. He also included those colonies in which it was not possible to determine with certainty

---

* Pacini, A. J. P., and Russell, D. W. Jour. Biol. Chem., xxxiv, 1918, 43.

whether the growth came from one or more cells, but t ere was no exclusion because of irregularities in growth.

Following this technique he made several seedings of yeast cells on the control solution after adding various substances, the influence of which on the growth of yeast it was desired to study. In Williams' studies he employed "protein free milk", alcoholic extract of "protein free milk", alcoholic extract of wheat germ, lactose extracted with alcohol, pancreas tissue, hydrolyzed casein, malt wort absorbed by Fullers earth, etc. As an illustration of the outcome of such experiments the following tables from Williams' paper are reproduced.

Williams' Table IV

1. 25 cc control solution 1 cc H_2O	14	8	10	4	5.4
2. 25 cc control solution 1 cc ether extract pancreatin	13	6	15	5	9.2
3. 25 cc control solution 1 cc alcohol extract pancreatin	15	13	280	80	187

Table XII shows typical results which were obtained in experiments designed to show the influence of heat on the antineuritic substance as its value is effective for the nutrition of yeast.

Williams' Table XII

	Average Growth After 6 Hours	Average Maxima After 23 Hours
1. 25 cc conc. sol. + 6 cc H_2O	3.3	9
2. 25 cc con. sol. + 6 cc autoclaved solution	4.3	163
3. 25 cc con. sol. + 6 cc unautoclaved extract	5.6	307

These tables show that there is a great difference between the growth of yeast in the control solution and in the others, and Williams believed that the substance which promotes growth was partially destroyed by heating 30 minutes under 15 lbs. pressure.

Williams does not give detailed results for his experiments on the treatment of preparations with acid or alkali, but makes the statement that after such treatment the material is changed presumably by hydrolysis to a form which is more immediately available to the yeast. Notwithstanding the fact that the total amount of yeast growth in two or three days is unaltered, the initial growth which takes place in the first 24 hours may be three times as great as in experiments with unhydrolyzed material.

Williams also tested the value of the dietary factor fat-soluble A on the growth of yeast. An alcoholic extract of egg yolk was prepared, and from the residue an ether extract was made. The average growth of yeast on the control solution was 4.5, whereas on the addition of 1 cc of the ether extract of egg yolk to 25 cc control solution the average growth was 9.0. On addition of 1 cc of the alcoholic extract of egg yolk growth was 68.0. On

addition of alcoholic extract plus ether extract the growth was 59.0. From these results Williams reached the conclusion that the ether extract, which would be rich in fat-soluble A but poor in water-soluble B, has no special value in promoting the growth of yeast, and therefore that the fat-soluble A is without consequence in the growth of yeast. However, by adding an alcoholic extract of egg yolk, which would furnish among other things the substance water-soluble B, growth was markedly stimulated.

The work reported in this paper had its origin in an effort to develop proficiency in the conduct of experiments with yeast by the Williams method.

Considerable inconvenience was experienced in carrying out the test, owing to the number of small particles which became detached from the pen, and which interfered with the counting of the number of cells. A modification of Williams' method was therefore introduced, in which a platinum syringe needle, having an opening at right angles to its axis, was used for depositing the droplets on the cover slip. The needle was attached to a piece of glass tubing by means of a short piece of rubber tubing, and it was found that with this instrument the suspension of yeast cells could be easily and quickly distributed, without leaving any detritus in the droplets. It was found that a fairly uniform distribution of cells could be obtained by this method. The slides were incubated in the manner described by Williams.

Our results were characterized by lack of uniformity in the development of the yeast, and for this reason an attempt was made to substitute for the cover slips used by Williams the ordinary blood counting chamber of the Levy type. In order to break up the clumps of yeast cells without injuring their vitality air was blown through the suspension instead of shaking the latter. The solution was then distributed in test tubes, 5 or 10 cc in each. The results by this technique were much more uniform. Table II shows on one hand how the cells

frequently were distributed in the droplet method, and on the other how the same suspension behaved in the blood counting chamber method.

Williams called attention in his paper to the fact that many times it is impossible to count the number of cells after a period of growth on account of the fact that the cells grow in three dimensions. He found it necessary to count a part and estimate the probable number. By the counting chamber method it is not necessary to guess at the number of cells. If the suspension is too rich it may be diluted to such a point as facilitates the counting. Immediately before counting a little formalin was added to the solution in order to kill the cells and avoid further growth. Using this technique we have attempted to test several substances known to be rich or poor in the dietary factor water-soluble B. We will first consider the results obtained by the extraction of wheat germ with hot water.

1 gm. of wheat germ was boiled with 50 cc of water for 10 minutes (extract 1) the residue was boiled again with 50 cc of water for 10 minutes (extract 2), and the second residue was boiled with 50 cc of water for 10 minutes (extract 3). The separation of the residue from the soluble part offered some difficulty due to the viscosity of the solution. In some cases we have separated the solution from the undissolved part by means of a centrifuge and in others by filtration in an alundum crucible. Only by passing the solution through a Berkefeld filter could an approximately clear solution be obtained. Eight successive extractions were made.

These extracts were evaporated to the volume of 25 cc, so each 1 cc corresponded to 0.04 gm . of wheat germ. 5 cc of the control solution was put into test tubes previously sterilized, and the first extract was added as shown in the following table.

Table I

Tubes	Control Sol.	1st. Extract	Control Sol.
1	5.0	0 cc	Seeded with
2	5.0	.1 "	Fleischman's
3	5.0	.2 "	Yeast
4	5.0	.3 "	
5	5.0	1.0 "	

From these tubes we made a series of slides by the droplet method and ran a comparative series by the counting chamber method, the results of which are presented in the following table.

Table II

Before Incubation

Tube #1	Tube #2	Tube #3	Tube #4	Tube #5
1 2 1 0 0	0 1 2 2 1	1 2 0	2 1 0 0	1 1 1 0 0
1 3 0² 0 0	3 2 1² 3 2	2 1 3 2 0	3 2 2 1	0 2 1 3 0
1 2 2 1² 1	1 1 1 0² 2	2 2 0 0	2 1 2 1	0 2 3
1 1 1	2 2 0 1	2 3 0 2	3 0 1 0² 1	0 0 2 1
0 1 1 1	0 3 2 1²	2 2	4 0 1 0 0	2² 1 2 0

After Incubation for 18 Hours

1 500 0 0 0	0 1 0 130 0	20 252 30 0 0	2 1 2 0 0	0 500 0
27 3 2 0 0	0 2 143 6 98	238 0 0 0	0 2 500 0	0 5
1 2 0 0 0	0 284 143	0 2000 0 0 0	2 1 0 1000	0 342 500
19 3 0	3 2 31 0 0	2 290 0 1000	440 0 500	0 0 0
0 2 3	0 0 4 0	2 374 0 300	1000 0	0

If we take into account only those droplets which contain one single cell, we observe that they give rise to very different numbers of cells by the end of an 18 hour incubation period. In those squares where the yeast cell was dead, or had reached the stage where budding had ceased, there was, of course, no multiplication.

Seeding #1 One cell gave rise to 3 cells
" " " " " 14 "
" " " " " 27 "
" " " " " 34 "
" " " " " 62 "

Seeding #2 (control solution plus 0.1 cc of wheat germ extract)

One cell gave rise to 3 cells
In the rest the single cells were dead or incapable of budding

Seeding #3 One cell gave rise to 101 to 301 cells
Two cells gave rise to 252 to 2000 cells

Seeding #4 One cell gave rise to 130 to 1000 cells.

Seeding #5 One cell gave rise to 75 to 1000cells

On the other hand, when using the test tubes which contain 5 to 10 cc of control solution, to which different amounts of wheat germ extract were added as stated above, by using the counting chamber we have a greater chance of securing the same number of cells distributed evenly, as respects their vital conditions, and thus effect a diminution of the magnitude of error due to individual cell differences. This procedure, in fact, gave more comparable results.

By the counting chamber method, and using the same suspension already used with the droplet method, the results tabulated in the following table were secured.

Table III

Tubes	First Count	Second Count (After 18 Hrs)	Average
1	21 cells	23 cells	22 cells
2	132 "	92 "	112 "
3	214 "	198 "	206 "
4	342 "	308 "	325 "
5	602 "	684 "	648 "

The largest variation in the series of countings on the same suspension was in tube 5 (602 to 684 - difference 82, about 13% variation between the first and second counting).

It seems, therefore, that a more even and reliable result can be obtained by the use of this procedure than with the droplet method devised by Williams, even though the droplets were made by the platinum needle instead of a pen.

We have tested the effect of increasing the quantity of the first aqueous extraction of wheat germ on the growth of yeast. The uniformly beneficial effects were suggestive that the high content of water-soluble B in the extract exerted a stimulating effect on the growth of the yeast.

The results of testing the second and third extracts prepared from the residue of the first extraction when tested for their effect on the growth of yeast are shown below. The results with the second extract are tabulated in Table IV, those with the third extract in Table V.

Table IV

	Number of Cells	Averages after 12 hr. incubation
Control solution	21	21
" " + .1 cc	132 and 92	112
" " + .2 cc	214 " 198	206
" " + .4 cc	342 " 308	325
" " +1.0 cc	602 " 684	648

Table V

	Number of Cells	Averages after 24 hr. Incubation
Control solution	150 and 189	118.5
" " + 1.0 cc	378 " 366	372
" " + 2.0 cc	421 " 480	450.5
" " + 3.0 cc	511 " 554	532.5
" " + 1.0 cc 1st. ext.	1068 " 1240	1154
" " + 1.0 cc 2nd. ext.	649 " 633	641

The results show clearly that there is a stimulating effect of the extracts on the rate of multiplication of yeast and that in general the greater the amount of extract added the more pronounced the acceleration.

Alcoholic extracts of wheat germ were also prepared in which various strengths of alcohol were used, varying from 40 to 75%. In all cases the germ was boiled for 10 minutes in alcohol and the solution filtered through paper or an alundum filter, then evaporated on a water bath at low temperature with a fan blowing on the surface. In all cases it was observed that the growth of yeast was markedly stimulated by the addition of an alcoholic extract of wheat germ to the control solution.

In some experiments we have used wheat germ extract treated with sodium carbonate and autoclaved, in order to destroy as much as possible of the antineuritic substance. Before addition of such extracts to the nutrient medium the sodium carbonate was neutralized with hydrochloric acid.

Extract of fresh beef made by boiling the latter with water was also employed. The beef muscle was treated with 2 per cent of $NaHCO_3$, moistened and allowed to stand for 3 hours, then autoclaved one hour at 15 lbs. pressure. Tests of this meat on rats showed it to be ineffective for the relief of polyneuritis. Extract of rolled oats was also used. The oats were treated

with 2 per cent of $NaHCO_3$ and autoclaved at 15 lbs. pressure as described for the meat muscle. The oats so treated were fed to young rats in a diet consisting of rolled oats 40.0, gelatin 5.0, casein 5.0, NaCl 1.0, butter-fat 3.0, $CaCO_3$ 1.5 per cent. The animals failed to grow. After 41 days 3 per cent of untreated wheat germ was added to the diet to furnish the antineuritic substance. The animals responded at once with rapid growth. 40 per cent of untreated oats furnish an abundance of antineuritic substance, water-soluble B, for growth. The treatment of the oats with alkali had, therefore, destroyed its content of this substance.

The series has been extended by the employment of meat extract prepared by precipitation with barium, the addition of barium being stopped while the solution was still distinctly acid. After the removal of the barium sulphate the remaining acidity was neutralized by ammonium hydroxide.

All extracts employed in our experiments were carefully sterilized by boiling and kept in an ice-box until used. Observations were made to establish that there was no contamination of these extracts with bacteria.

Table VI illustrates the effect of ~~varying the quantity of~~ 40% alcoholic extract of wheat germ added to the control solution on the rate of development of yeast cells. Equal amounts of successive extracts were employed.

Table VI

	No. of Cells After 18 Hrs.	Averages
5 cc of control solution	147 and 133	142.5
" " " " " + 1 cc 1st ext.	811 " 798	804.5
" " " " " + 1 cc 2nd ext.	507 " 509	508
" " " " " + 1 cc 3rd ext.	392 " 416	404
" " " " " + 1 cc 4th ext.	430 " 399	409.5

Table VII illustrates the effect of adding 1 cc of each of a series of eight successive extracts of wheat germ, using 40 per cent ~~of~~ alcohol as a solvent.

Table VII

	No. of Cells After 20 Hours			Averages
5 cc control solution	570	433	480	494.5
" " " " + 1 cc 1st ext.	1560	1720		1640
" " " " + 1 cc 2nd "	1610	1720		1500
" " " " + 1 cc 3rd "	890	810		850
" " " " + 1 cc 4th "	760	670		765
" " " " + 1 cc 5th "	590	690		640
" " " " + 1 cc 6th "	820	680	780	760
" " " " + 1 cc 7th "	760	560		660
" " " " + 1 cc 8th "	500	600		555

Table VIII illustrates clearly the progressively increasing benefit to the yeast by adding increasing amounts of 75 per cent alcohol extract of wheat germ.

Table VIII

	No. Cells After 20 hrs. inc. 400 squares		Averages
5 cc control solution + 1 cc H_2O	499	483	491
" " " " + .9 cc " + .1 cc lst ext.	1000	870	935
" " " " + .8 cc " + . 2 cc " "	1360	1390	1325
" " " " + .6 cc " + . 4 cc " "	1610	1740	1670
" " " " +1.0 cc " + 1.0 cc " "	2180	2540	2360

Tables IX, X, and XI illustrate the effect of adding to the control solution aqueous or alcoholic (40 per cent alcohol) extracts of wheat germ. In

these experiments the wheat germ had been treated with 2 or 4 per cent sodium bicarbonate, allowed to stand in a moist condition for three hours or longer, in order that the alcohol might penetrate the material. The wheat germ was then autoclaved at 15 lbs. pressure for three hours, the acid neutralized and the material dried in a current of warm air.*

Table IX

	No. Cells After 18 Hours Per 400 Squares		Averages
5 cc control solution	770	580	625
" " " " + 1 cc autoclaved wheat germ ext.	2460	1860	2160
" " " " + 2 cc " " " "	2650	3530	3090
" " " " + 1 cc alch. ext. wheat germ	2520	2420	2470
" " " " + 2 cc " " " "	2530	2320	2425

Table X

	No. Cells After 18 Hours Per 400 Squares		Averages
5 cc control solution	160	210	185
" " " " + 1.5 cc H_2O + .5 cc 75% alc. ext.	1340	1210	1275
" " " " + 1 cc " + 1 cc " " "	2060	2030	2045
" " " " + 1 cc " + .5 cc " " " + 1 cc autocl. wheat germ	1920	1480	1700
" " " " + 1 cc autoclaved wheat germ + 1 cc 75% alc. ext.	2790	3920	2905
" " " " + 1 cc autoclaved wheat germ + 1 cc H_2O	2190	2120	2155

---

* This germ was used in a feeding experiment with rats to show whether it still contained any appreciable amount of water-soluble B. The ration consisted of casein 18, treated wheat germ 15, butter-fat 5, salt mixture (185) 3.7, dextrin 58.3. The animals failed to grow. In a subsequent period rapid growth took place when 3 per cent of untreated germ was included in the diet. The antineuritic substance had been practically all destroyed by heating with alkali.

Table XI

	No. Cells After 20 Hrs per 400 Sq.		Ave.
5 cc cont. sol. + 2 cc H_2O	100	120	110
" " " " + 1 cc " + 1 cc ext. autocl. wheat germ	2440	2320	2280
" " " " + 2 cc " " " "	2730	2630	2680
" " " " + 1 cc " + 1 cc 40% alc. ext. " "	2040	2320	2180
" " " " + 2 cc " " " " "	2190	1670	1915

It is easily apparent that the addition of these extracts exerted a profound stimulating effect on the growth of the yeast, but that the extent of acceleration of the growth of yeast is not proportional to the amount of extract added.

Table XII illustrates the effect on the growth of yeast of adding to the control solution varying amounts of glucose and of glucose together with extracts of wheat germ.

Table XII

	24 Hours		48 Hours	
	Counts	Ave	Counts	Ave
1. 10 cc control sol.	(300)310	305	(2350)2500	2425
2. " " " " + 1 cc glucose 10%	(390)330	360	(5020)4940	4980
3. " " " " + 2 cc " " (	360) 380	370	(8300) 7650	7975
4. " " " " + 1 cc autocl. wheat germ ext.	(1540)1350	1445	(2880)3060	2970
5. " " " " + 1 cc autocl. wheat germ ext. + 1 cc glucose 10%	(1400)1390	1395	(3780)4020	3900
6. " " " " + 1 cc 75% alco. ext. wheat	(1650)1810	1730	(5340)5450	5395
7. " " " " + 1 cc 75% alco. ext. wheat + 1 cc glucose 10%	(1570)1540	1555	(5400 (5760	5580

It will be observed that numbers 2 and 3 show a surprising acceleration due to the addition of double the amount of glucose. 1 cc of 10% glucose solution added to 10 cc of control solution produced 4980 yeast cells after 48 hours. 2 cc of the glucose solution added to 10 cc of control solution led to the development of 7975 cells in the same interval. Comparing 4 and 5, the addition of 1 cc of autoclaved wheat germ extract produced 2970 cells in 48 hours, whereas the same medium with 1 cc of 10% solution produced 3900 cells. That results are not always consistent is shown by comparison of 6 and 7, in which there was a marked effect from the addition of 1 cc of 75% alcoholic extract of wheat germ, and very slight further stimulation by the addition of 1 cc of 10% glucose.

Table XIII illustrates further the lack of uniformity in the development of yeast under different conditions.

Table XIII

	18 Hrs Counts	18 Hrs Aver.	18 hrs. Counts	18 hrs. Aver.
1. 10 cc cont. sol.	(350) 290	320	(4680) 4100	4390
2. " " " " + 1 cc glocuse 10%	(420) 360	400	(4660) 4620	4640
3. " " " " + .1 cc 75% al. ext. wheat germ.	(620) 670	645	(3600) 3760	3680
4. " " " " + " " " " " " " + 1 cc glucose 10%	(910) 980	945	(6260) 5320	5790
5. " " " " + .5 cc 75% alc. ext. wheat germ	(940)1300	1120	(4240) 4400	4320
6. " " " " + " " " " " " " + 1 cc glucose 10%	(930) 890	910	(4340) 4452	4395
7. " " " " + 1 cc 75% alc. ext. wheat germ	(1250)1800	1525	(4420) 4590	4505
8. " " " " + " " " " " " " + 1 cc glucose 10%	(1130 (1240	1185	(4380) 4190	4285

Comparing 2 and 3, greater growth was secured by adding to 10 cc of the control solution 1 cc of 10% glucose solution than was obtained by the addition of 0.1 cc 75% alcoholic extract of wheat germ without additional glucose. In order to test the possibility of securing comparable development of yeast when the cultural conditions for every cell were as nearly as possible identical the following procedure was tried.

A drop of yeast suspension was placed on a clean cover slip, and the number of yeast cells contained in it carefully determined by counting. The yeast cells were then immediately washed into a sterile test tube by means of control solution, and the amount of control solution then adjusted so that 0.1 cc of nutrient solution was available for each yeast cell present. The following five experiments illustrate the results of this procedure. An examination of Table XIV shows that in the first test tube there were 21 yeast cells, each with 0.1 cc of nutrient medium. After 12 hours the number was increased to 39, an approximate multiplication of the original number by 2. In the second tube 29 cells were present, and in 12 hours this had multiplied the original number by 16. In the third tube 31 cells were present, and after 12 hours this had multiplied the original number by 19. In the fourth tube 34 cells were present, and after 12 hours this had multiplied 21 1/2 times the original number. In the fifth tube 43 cells were present, and in 12 hours the original number was multiplied by 10. The cause of these irregular results is unquestionably to be attributed to the introduction of injured cells or cells of low reproductive power into the test specimens.

Table XIV

			After 12 Hours	Average
1st tube	21 cells	2.1 cc cont. sol.	43 35	39
2nd "	29 "	2.9 " " "	464 461	463.5
3rd "	31 "	3.1 " " "	594 586	590
4th "	34 "	3.4 " " "	767 698	732.5
5th "	43 "	4.3 " " "	422 454	438

We have sought to test the value of a procedure involving the addition of 1 drop of opalescent suspension of yeast cells in water to several test tubes, each containing 10 cc of nutrient solution, and then counting these cells in the Levy chamber before incubation.

Table XV illustrates one set of experiments in which fairly close agreement was obtained by this procedure, but the agreement is presumably due to the good fortune of selecting a series of cells all or nearly all of which were capable of development.

Table XV

		After 22 Hrs	Average
Tube 1	3 cells per 400 square	186 and 198	192
" 2	4 " " " "	174 and 195	184.5
" 3	3.5 " " " "	169 and 153	161

In Table XVI are recorded the results of a series of trials designed to determine the effect of various extracts on the rate of development of yeast. The volume of solution was kept constant by the addition of glucose solution or water. The materials from which the extracts were prepared in these experiments had been subjected to heat under pressure in the presence of sodium bicarbonate as described in the methods of preparation of materials. In all cases these preparations had been tested with rats and found to be at least practically free from the dietary factor water-soluble B. Notwithstanding the fact that they contained so little of this substance as to be undemonstrable by experiments on the rat the extracts were still very effective in stimulating the growth of yeast. These results suggest that although the addition of the antineuritic substance in minute amounts may have influenced the growth of yeast in these experiments, this factor is so small as to make it practically certain that other added substances, as glucose and amino-acids, also served to influence the results in a marked way.

Table XVII illustrates the results obtained in a series of experiments in which it was sought to estimate by the gas produced by the yeast culture the extent of growth of the yeast in several types of modified nutrient medium. These results are so variable when we compare the gas production with the number of yeast cells generated during the period of incubation, that the measurement of the amount of gas produced does not give promise of value in this line of work.

In Table XVIII are presented a series of observations on the effect of adding an amino-acid mixture obtained by the hydrolysis of beef muscle with sulphuric acid.

Table XVI

	After 18 Hours	Averages
1. 9 cc control solution 1 cc 10% glucose solution 1 cc H_2O	390 and 350	370
2. 8 cc control solution 2 cc glucose solution 1 cc H_2O	310 " 380	345
3. 8 cc control solution 2 cc glucose solution 1 cc 8th extract wheat germ	320 " 340	330
4. 8 cc control solution 2 cc dextrose solution 1 cc autoclaved wheat ext. (1st)	1750 " 1610	1680
5. 8 cc control solution 2 cc glucose solution .5 cc 1st extract wheat germ .5 cc H_2O	1880 " 1750	1815
6. 8 cc control solution 2 cc glucose solution 1 cc 1st extract wheat germ	1960 " 1990	1975
7. 8 cc control solution 2 cc glucose solution 1 cc rolled oats extract (made from rol ed oats autoclaved with 2% $NaHCO_3$)	1980 " 2010	1995
8. 8 cc control solution 2 cc glucose solution 1 cc beef extract (from b ef steak autoclaved with 2% $NaHCO_3$	1700 " 1760	1730

Table XVII

	After 20 Hours		After 43 Hours		Gas
	Counts	Aver.	Counts	Aver.	cc.
1. 9 cc control solution 1 cc 10% glucose solution 1 cc H_2O	(156) 137	146.5	(2510)2880	2685	2.5
2. 8 cc control solution 2 cc glucose solution 1 cc H_2O	(143) 139	141	(2300)2180	2240	2.5
3. 8 cc control solution 2 cc glucose solution 1 cc 8th ext. wheat germ	(133) 128	130.5	(1740)2010	1375	.5
4. 8 cc control solution 2 cc glucose solution 1 cc autocl. wheat extract (1st)	(732)	732	(4180)4230	4205	10
5. 8 cc control solution 2 cc glucose solution .1 cc 1st. ext. wheat germ	(493) 535	514	(3900)4780	4340	4
6. 8 cc control solution 2 cc glucose solution .5 cc 1st. ext. wheat (alcohol) .5 cc H_2O	(675)		(5020)5210	5115	5
7. 8 cc control solution 2 cc glucose solution 1 cc 1st ext. wheat germ (alco.)	(1340)		(4740)4100	4420	8
8. 8 cc control solution 2 cc glucose solution 1 cc 5th ext. wheat germ (alco.)	(170) 143	156.5	(4830)5140	4985	
9. 8 cc control solution 2 cc glucose solution 1 cc ext. rolled oats (fresh oats)	(636) 752	692	(3680)4310	3995	8
10. 8 cc control solution 2 cc glucose solution 1 cc steak extract (alkalinized steak)	(1027)		(4560)4940	4750	10
11. 8 cc control solution 2 cc glucose solution 1 cc 3rd ext. wheat germ (alco.)	(880)		(2230)2340	2285	3

Table XVIII

	After 20 Hours	Averages
1. 1 cc control solution	1020 and 1230	1125
2. 1 cc " " 1 cc autoclaved wheat extract	1980 " 2080	2030
3. 10 cc control solution .5 cc digested meat (prepared from fresh steak not autoclaved with alkali)	1820 " 1970	1895
4. 10 cc control solution .5 cc digested meat extract (prepared from fresh steak not autoclaved with alkali)	2048 " 2200	2124
5. 10 cc control solution 1 cc digested meat extract (prepared from fresh steak not autoclaved with alkali)	2412 " 1940	2176
6. 10 cc control solution 1 cc 1st. ext. wheat germ	2020 " 2380	2200
7. 10 cc control solution .5 cc 1st ext. wheat germ	1680 " 1880	1780

So far as our knowledge permits us to judge, these digested meat extracts could serve only as a source of amino-acids, since the treatment was sufficient to reduce the proteins practically to the amine-acid state, and since muscle tissue is even in the fresh condition very poor in the antineuritic factor (water-soluble B), the same meat after hydrolysis with strong sulphuric acid would scarcely contain a trace of it. Yet these meat digests exerted a profound influence on the rate of development of yeast, approximately doubling the number of cells which were counted after 20 hours incubation in experiments 6 and 7, Table XVIII. It is again apparent that doubling the amount of the

antineuritic substance derived from wheat germ extract does not exert a proportional stimulating effect on the growth of yeast.

The series of observations which we have presented above seem to us to form a conclusive demonstration that the use of yeast as a test organism for determining the presence or absence of the antineuritic dietary factory is complicated by so many disturbing factors as to make it of little if any value. Whenever extracts of natural foods are to be tested it is inevitable that food substances of one kind or another which greatly stimulate the growth of yeast must be added simultaneously with the unidentified dietary essential for which the test was designed. Glucose and amino-acids effect such stimulation.

We do not desire to draw the deduction from the data here presented that the antineuritic substance is not necessary for the development of yeast, or that it does not stimulate the growth of yeast, but the several modifications of the procedure originally described for the utilization of yeasts as a test for the antineuritic substance point so definitely to several difficulties in the way of making this a satisfactory test that it has seemed to us desirable to present the results for the benefit of those who may be interested in this line of work.